Supplementary Appendix

Supplement to: Takashita E, Kinoshita N, Yamayoshi S, et al. Efficacy of antibodies and antiviral drugs against Covid-19 omicron variant. N Engl J Med. DOI: 10.1056/NEJMc2119407

This appendix has been provided by the authors to give readers additional information about the work.

Supplementary Appendix

Contents

Materials

Cells. VeroE6/TMPRSS2 (JCRB 1819) cells were propagated in the presence of 1 mg/ml geneticin (G418; Invivogen) and 5 μg/ml plasmocin prophylactic (Invivogen) in DMEM containing 10% FCS and antibiotics. The cells were incubated at 37 °C with 5% CO_2, regularly tested for mycoplasma contamination by using PCR, and confirmed to be mycoplasma-free.

Clinical specimens. After informed consent was obtained, specimens were collected from individuals with SARS-CoV-2 infection. The research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science of the University of Tokyo (approval number 2019–71–0201).

Viruses. hCoV-19/Japan/NC928-2N/2021 (Omicron; NC928), SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo (NCGM02), SARS-CoV-2/UT-HP127-1Nf/Human/2021/Tokyo (Alpha; HP127), hCoV-19/USA/MD-HP01542/2021 (Beta; HP01542), hCoV-19/Japan/TY7-503/2021 (Gamma; TY7-503), and hCoV-19/USA/WI-UW-5250/2021 (Delta; UW5250)] were propagated in VeroE6/TMPRSS2 cells in VP-SFM (Thermo Fisher Scientific).

All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Tokyo and the National Institute of Infectious Diseases, Japan, which are approved for such use by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Antibodies. Amino acid sequences for the variable region of the heavy and light chains of the following human monoclonal antibodies against the S protein were used for gene synthesis: clones etesevimab (CB-6/LY-CoV016; accession # QJU69682 and QJU69681), tixagevimaba (COV2-2196/AZD8895; QLI33947 and QLI33948), casirivimabb (REGN10933; 6XDG_B and 6XDG_D), cilgavimaba (COV2-2130/AZD1061; QKY76296 and QKY75909), bamlanivimab (LY-CoV555; 7KMG_A and 7KMG_B), imdevimabb (REGN10987; 6XDG_A and 6XDG_A), and sotrovimab (S309/VIR-7831; 6WS6_A and 6WS6_F). An artificial signal sequence and the constant gamma heavy (IgG1, accession # P01857) and kappa (accession # P01834) or lambda (accession # P0DOY2) light chain coding sequences were added before and after each variable region. Codon usage was optimized for expression in CHO cells. The synthesized genes were cloned into a plasmid for protein expression and transfected into CHO cells. Cell culture media were harvested after incubation for 10–14 days at 37 °C and human monoclonal antibodies were purified using MabSelect SuRe LX (Cytiva). Purity was confirmed by SDS-PAGE and HPLC before use.

Antiviral compounds

Active components of remdesivir, molnupiravir, and PF-07304814 (i.e., GS-441524, EIDD-1931, and PF-00835231) were purchased from MedChemExpress. All compounds were dissolved in dimethyl sulfoxide.

Methods

Whole genome sequencing.

Viral RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN). The whole genome of SARS-CoV-2 was amplified by using a modified ARTIC network protocol in which some primers were replaced or added (ref 1, 2). Briefly, viral cDNA was synthesized from the extracted RNA by using a LunarScript RT SuperMix Kit (New England BioLabs). The DNA was amplified by multiplexed PCR in two pools using the ARTIC-N1 primers v4 or v5 (ref 3) and the Q5 Hot Start DNA polymerase (New England BioLabs). The DNA libraries for Illumina NGS were prepared from pooled amplicons by using a QIAseq FX DNA Library Kit (QIAGEN) and were then analyzed by using the iSeq 100 System (Illumina). The reads were assembled by the CLC Genomics Workbench (version 21, Qiagen) with the Wuhan/Hu-1/2019 sequence (GenBank accession no. MN908947) as a reference. The sequence of hCoV-19/Japan/NC928-2N/2021 (Omicron; NC928) was deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database (ref 4) with Accession ID: EPI_ISL_7507055.

Enzyme-Linked Immunosorbent Assay (ELISA).

Ninety-six-well Maxisorp microplates (Nunc) were incubated with the prefusion-stabilized spike variant, termed HexaPro for its six stabilizing proline substitutions (50 μl/well at 2 μg/ml), or with PBS at 4 °C overnight and were then incubated with 5% skim milk in PBS containing 0.05% Tween-20 (PBS-T) for 1 h at room temperature. The microplates were reacted for 1 h at room temperature with the monoclonal

antibodies that were serially 2-fold diluted in PBS-T containing 5% skim milk, followed by peroxidase-conjugated goat anti-human IgG, Fcγ Fragment specific antibody (Jackson Immuno-Research) for 1 h at room temperature. 1-Step Ultra TMB-Blotting Solution (Thermo fisher scientific) was then added to each well and incubated for 3 min at room temperature. The reaction was stopped by the addition of 2 M H_2SO_4 and the optical density at 450 nm (OD_{450}) was immediately measured. The OD_{450} values of the PBS-containing wells were subtracted from the OD_{450} values of the HexaPro-containing wells for background correction. A subtracted OD_{450} value of 0.1 or more was regarded as positive; the minimum concentration to give a positive result was used as the ELISA titer.

Focus reduction neutralization assay (FRNT)

Neutralization activities of SARS-CoV-2 were determined by using a focus reduction neutralization assay as previously described (5). Serial dilutions of monoclonal antibodies (starting concentration, 50,000 ng/ml) were mixed with 1000 focus-forming units (FFU) of virus/well and incubated for 1 h at 37 °C. The antibody-virus mixture was inoculated on VeroE6/TMPRSS2 cells in 96-well plates in duplicate and incubated for 1 h at 37 °C. An equal volume of 1.2% Avicel RC-581 (DuPont Nutrition USA) in culture medium was added to each well. The cells were incubated for 24 h at 37 °C and then fixed with formalin. After the formalin was removed, the cells were immunostained with a mouse monoclonal antibody against SARS-CoV-1/2 nucleoprotein [clone 1C7C7 (Sigma-Aldrich)], followed by a horseradish peroxidase-labeled goat anti-mouse immunoglobulin (SeraCare Life Sciences). The

infected cells were stained with TrueBlue Substrate (SeraCare Life Sciences) and then washed with distilled water. After cell drying, the focus numbers were quantified by using an ImmunoSpot S6 Analyzer, ImmunoCapture software, and BioSpot software (Cellular Technology). The results are expressed as the 50% focus reduction neutralization titer ($FRNT_{50}$). The $FRNT_{50}$ values were calculated by using GraphPad Prism (GraphPad Software).

Inhibitory effect of compounds against SARS-CoV-2 *in vitro.*

Antiviral susceptibilities of SARS-CoV-2 were determined by applying a focus reduction assay as previously reported for influenza virus (6). VeroE6/TMPRSS2 cells in 96-well plates were infected with 1000 FFU of virus/well. Virus adsorption was carried out for 1 h at 37 °C and then an equal volume of 1.2% Avicel RC-581 (DuPont Nutrition USA) in culture medium containing serial dilutions of antiviral compounds was added to each well in triplicate. The cells were incubated for 24 h at 37 °C and then fixed with formalin. After the formalin was removed, immunostaining was performed as described for the FRNT. The results are expressed as the 50% inhibitory concentration (IC_{50}). The IC_{50} values were calculated by using GraphPad Prism (GraphPad Software).

Table S1. Amino Acid Substitutions in the hCoV-19/Japan/NC928-2N/2021 stock virus used in this study.*

Gene	hCoV-19/Japan/NC928-2N/2021
ORF1a	Lys856Arg, Pro1803Leu, Thr1822Ile, Ser2083del, Leu2084Ile, Ala2710Thr, Thr3255Ile, Pro3395Tyr, Leu3674del, Ser3675del, Gly3676del, Ile3758Val, Leu3829Leu(58%)/Phe(42%)
ORF1b	Pro314Leu, Ile1566Val
S†	Ala67Val, His69del, Val70del, Thr95Ile, Gly142Asp Val143del, Tyr144del, Tyr145del, Asn211del, Leu212Ile, Arg214^Asp215_ins_GluProGlu, **Gly339Asp, Ser371Leu, Ser373Pro, Ser375Phe, Lys417Asn, Asn440Lys, Gly446Ser, Ser477Asn, Thr478Lys, Glu484Ala, Gln493Arg, Gly496Ser, Gln498Arg, Asn501Tyr, Tyr505His,** Thr547Lys, Asp614Gly, His655Tyr, Asn679Lys, Pro681His, Asn764Lys, Asp796Tyr, Asn856Lys, Gln954His, Asn969Lys, Leu981Phe
E	Thr9Ile
M	Asp3Gly, Gln19Glu, Ala63Thr
ORF9b	Pro10Ser, Glu27del, Asn28del, Ala29del
N	Pro13Leu, Glu31del, Arg32del, Ser33del, Arg203Lys, Gly204Arg

*Substitutions based on a comparison with the Wuhan/Hu-1/2019 sequence.

†Substitutions in the Receptor Binding Domain of S are indicated in boldface type.

Table S2. Reactivity of monoclonal antibodies with different SARS-CoV-2 variants

SARS-CoV-2 variant	ELISA titer (ng/ml)*						
	Etesevimab (CB-6/LY-CoV016)	Bamlanivimab (LY-CoV555)	Imdevimab (REGN10987)	Casirivimab (REGN10933)	Tixagevimab (COV2-2196/AZD8895)	Cilgavimab (COV2-2130/AZD1061)	Sotrovimab (VIR-7831, S309)
Wuhan (A)	0.49	0.49	0.49	0.49	0.49	0.49	0.49
Alpha (B.1.1.7)	0.49	0.49	0.49	0.49	0.49	0.49	0.49
Beta (B.1.351)	>1000	500.00	0.49	0.49	0.49	0.49	0.49
Gamma (P.1)	1000	500.00	0.49	0.49	0.49	0.49	0.49
Delta (B.1.617.2)	0.49	3.90	0.49	0.49	0.49	0.49	0.49

*The minimum concentration to give a positive result is used as the ELISA titer.

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